KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas

Vincent T.H.B.M.Smit, Angelina J.M.Boot[1], Alida M.M.Smits[1], Gert Jan Fleuren, Cees J.Cornelisse and Johannes L.Bos [1*]

Department of Pathology and [1]Laboratory for Molecular Carcinogenesis, State University of Leiden, PO Box 9305, 2300 RA Leiden, The Netherlands

ABSTRACT
      DNAs from human pancreatic adenocarcinomas were analyzed for
the presence of mutations in codons 12,   13 and 61 of the   NRAS,
KRAS and HRAS gene. Formalin-fixed and paraffin-embedded   tissue
was used directly in an in vitro amplification reaction to expand
the relevant RAS sequences. The mutations were detected by
selective   hybridization   using   mutation-specific   synthetic
oligonucleotides. In 28 of the 30 patients we found a mutation in
codon 12 of the KRAS gene. This result confirms the findings of
Almoguera et al. [Cell 53 (1988) 549-554] that KRAS mutations
occur frequently in adenocarcinomas of the exocrine pancreas. The
mutations are predominantly G-T transversions, in contrast to the
KRAS mutations in colon tumors which are mainly G-A transitions.
Furthermore, in a portion of the tumors the mutation appears to
be homozygous.

INTRODUCTION
        Mutated RAS genes have been detected in a broad range of
human cancers [1]. For instance, half of the colon tumors [2-4]
and a third of the lung adenocarcinomas [5] and the acute myeloid
leukemias [6,7] harbor a mutated RAS gene. In all cases the
mutation was found in codon 12, 13 or 61 of one of the three
genes, NRAS, KRAS and HRAS, although there is some specificity in
that NRAS mutations predominate in hematopoietic malignancies and
KRAS mutations in colon tumors and lung adenocarcinomas.

        Tumors of the acinar cells of the exocrine pancreas are the
fourth common tumor in the western world and invariably result in
the patients death.   Recently, Almoguera and coworkers [8]
reported that mutations in or around codon 12 of the KRAS gene
occur in 21 of the 22 pancreas adenocarcinomas analyzed.   The
RNAse A mismatch cleavage procedure used, however, does not allow
the exact determination of the mutations. The knowledge of the
mutation is important to evaluate whether specific chemical

mutagens are involved in the mutational event. Furthermore, we would like to know whether tumors which do not contain a mutation in or around codon 12 of the KRAS gene may have a mutation in codon 61 of the KRAS gene or in one of the other RAS genes. We therefore analyzed a series of 30 pancreatic adenocarcinomas using our procedure of selective hybridization with synthetic oligonucleotides [9,10]. In this procedure we hybridize tumor DNA with oligonucleotides specific for mutations that can activate a normal RAS gene into an oncogene.

In this paper we confirm the high incidence of RAS mutations in pancreatic adenocarcinomas. The mutations were exclusively in codon 12 of the KRAS gene. The spectrum of mutations found is similar to the KRAS 12 mutations in lung adenocarcinomas but differs from the KRAS mutations in colon tumors. The results will be discussed in terms of possible chemical mutagens involved in the mutation of the KRAS gene.

MATERIAL AND METHODS

Tumor material

        Formalin-fixed, paraffin-embedded pancreatic carcinoma tissues were obtained from the University Hospital and the Diaconnessen Hospital, Leiden. The oldest samples were from 1973, the most recent from 1988. A section of paraffin blocks was stained with hematoxylin and eosin and examined for the presence of tumor cells. Three adjacent 10μM sections were taken and used in the analysis of mutated RAS gene.

In vitro amplification

        One 10μM section was used for each amplification. The amplification was essentially as described by Shibata et al. [11] with minor modifications. After deparaffination the tissue was suspended in 100μl H_2O and incubated for 10 min at 100° C. Subsequently, 20μl of a mixture was added containing buffer, dNTPs, primer and DNA polymerase. The final concentrations were: 50mM KCl, 10mM Tris.Cl pH8.3, 3mM $MgCl_2$, 0.01% bovine serum albumine, 200μM each dNTP, 1μM each primer and 2 units Taq-DNA polymerase (BIORES, Woerden, The Netherlands). The samples were cycled 40 times at 72°C, 94°C and 56°C, each for 1.5 min, using a robot-arm and three separate waterbaths. Subsequently, the specificity of the amplification was renewed by the addition of

Table I. Primers used for the polymerase chain reaction

	5′ site		3′ site	
outside primers:				
	-20	-1	111	92
KRAS 12	GGGAGAGAGGCCTGCTGAAA		CTCTATTGTTGGATCATATT	
HRAS 12	CCGCAGGCCCCTGAGGAGCG		CTCTATAGTGGGGTCGTATT	
NRAS 12	GAGGTTCTTGCTGGTGTGAA		CTCTATGGTGGGATCATATT	
	3	22	133	114
KRAS 61	TTCCTACAGGAAGCAAGTAG		ATACACAAAGAAAGCCCTCC	
HRAS 61	TTCCTACCGGAAGCAGGTGG		ACACACACAGGAAGCCCTCC	
NRAS 61	TTCTTACAGAAAACAAGTGG		ATACACAGAGGAAGCCTTCG	
inside primers:				
	3	22	68	49
KRAS 12	GACTGAATATAATCTTGTGG		AGCTGTATCGTCAAGGCACT	
HRAS 12	GACGGAATATAAGCTGGTGG		AGCTGGATGGTCAGCGCACT	
NRAS 12	GACTGAGTACAAACTGGTGG		AGCTGGATTGTCATGGCGCT	
	31	50	107	88
KRAS 61	GGAGAAACCTGTCTCTTGGA		CTCATGTACTGGTCCCTCAT	
HRAS 61	GGGGAGACGTGCCTGTTGGA		CGCATGTACTGGTCCCGCAT	
NRAS 61	GGTGAAACCTGTTTGTTGGA		CTCATGTATTGGTCTCTCAT	

new primers which nested between the first primers (1μM), dNTPs
(200μM each) and enzyme (2 units), and another 40 cycles were
performed. The primers used are shown in Table I. Normally, we
amplify the regions around codon 12 of the three RAS genes
simultaneously and, subsequently, the regions around codon 61.
For the pancreatic tumors we have first amplified the region
around codon 12 of the KRAS gene and only the samples negative
for mutations in codon 12 or 13 of the KRAS gene were amplified
for the other regions.

Selective hybridization

Selective hybridizations using oligonucleotide probes of 20
nucleotides length were performed as described by Verlaan-de
Vries et al. [10].

RESULTS

Thirty seven samples of formalin-fixed and paraffin-embedded tissue, most of which contained adenocarcinomas of the exocrine pancreas, were histologically characterized and the percentage of tumor cells in the sample was estimated. The samples were deparaffinized and relevant sequences of the three RAS genes were in vitro amplified by a procedure essentially as described by Shibata and coworkers [11]. We first amplified sequences comprising codons 12 and 13 of the KRAS gene. This in vitro amplified material was spotted onto 13 nylon membranes and hybridized to an oligomer complementary to the normal KRAS sequence in and around codon 12 and to 12 other oligomers complementary to all possible single point mutations that lead to an amino acid change in codon 12 or 13. Hybridization conditions were chosen so that only a fully matched hybrid remains. Only the autoradiograms that show positive hybridizations are shown in Figure 1. In examining these autoradiograms one should keep in mind that each tumor sample is independently amplified. Since the efficiency of amplification will vary, the amount of DNA spotted onto the membrane will also be variable. Therefore, one should not compare the intensity of the hybridization signal of the different tumor samples. Since the specific radioactivity of each of the oligomers is similar the different hybridization signals of an individual sample can be compared. In principle, the mutation-specific and the normal (gly) hybridization signal should be of equal intensity assuming that only one allele is mutated and that the sample does not contain normal tissue. Most of the samples, however, contain considerable amounts of normal tissue (see Table II) which should reduce the mutation-specific signal. In some cases a stronger mutation-specific signal is found (see for instance sample 22) indicating that the tumor cells have lost the normal KRAS allele. Finally, with some samples, a weak mutation-specific signal is observed in addition to a strong mutation-specific signal with another oligomer probe (e.g. samples 10, 16, 34, 36). This might suggest tumor heterogeneity, but the signals are too weak compared to the normal signal to draw firm conclusions. Our interpretation of the results is summarized in Table II. From the 37 samples 29 show a clear mutation-specific signal indicating that the corresponding
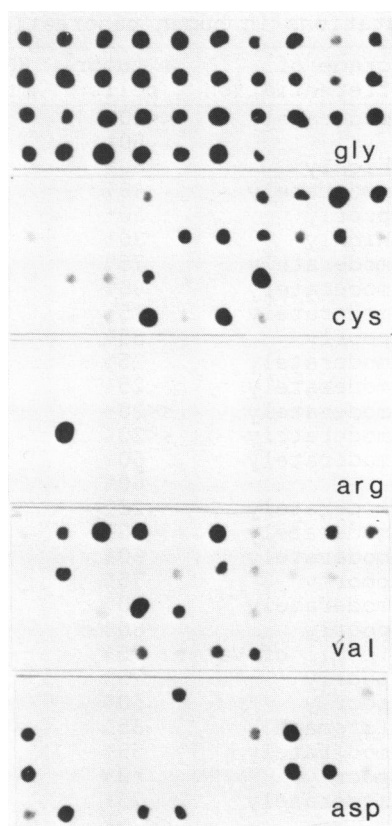
Figure 1. Demonstration and characterization of point mutations in codon 12 of the KRAS gene. Indicated are autoradiograms of hybridizations of identical dotblots to oligonucleotide probes specific for the normal sequence GGT of codon 12 (gly) and to mutated sequences TGT (cys), CGT (arg), GTT (val) and GAT (asp). The samples are spotted from left to right. First rows: samples 1-10, second rows: samples 11-20, third rows: samples 21-30 and fourth row: samples 31-37.

mutation is present in the tumor tissue. One of these positive samples was a repeat sample from the same patient. All the positive samples were pancreatic adenocarcinomas. Four samples did not contain sufficient tumor cells to allow detection by our procedure. In routine analyses we are able to detect a point mutation when it is present in 10-20% of the cells in the sample. Finally, four samples with sufficient tumor cells did not harbor a mutation in codon 12 or 13 of the KRAS gene. Only two of these

Table II. RAS mutations in human pancreatic adenocarcinomas

sample nr.	patient nr.	grade of differentiation	% tumor cells	KRAS 12 mutation	normal allele *
1	1	moderately	<<20%	-	
2			50%	val	
3	2	highly	60%	val	loss
4	3	moderately	40%	val	loss
5	4	poorly	50%	asp	
6	5	highly	30%	val	loss
7	6	moderately	75%	cys	
8	7	moderately	40%	cys	
9	8	moderately	75%	cys	loss
10	9	poorly	85%	cys	
11	10	moderately	25%	asp	
12	11	moderately	25%	val	
13	12	moderately	<<20%	-	
14	13	moderately	<<20%	-	
15	14	moderately	60%	cys	
16			60%	cys	
17	15	moderately	25%	cys	
18	16	moderately	80%	asp	
19	17	moderately	60%	cys	loss
20	18	poorly	75%	---	
21	19	moderately	50%	asp	
22	20	poorly	80%	arg	loss
23	21	(papil of Vater)	75%	---	
24	22	poorly	75%	val	
25	23	poorly	50%	val	
26	24	(stomach)	85%	---	
27	25	moderately	50%	cys	loss
28	26	poorly	50%	asp	
29	27	moderately	75%	asp	
30			<<20%	-	
31	28	moderately	30%	asp	
32	29	poorly	50%	asp	
33	30	poorly	50%	---	
34	31	moderately	50%	cys	
35	32	poorly	50%	asp	
36	33	highly	50%	cys	
37	34	poorly	75%	val	

-, not relevant; ---, no mutation detectable.
*, Only for clear cases the loss of the normal allele is indicated.

samples were pancreatic adenocarcinomas. The other two samples were a carcinoma of the papil of Vater and a stomach carcinoma, which were added as control samples. Thus, 28 of the 30 patients with an adenocarcinoma of the exocrine pancreas and with sufficient tumor cells in the sample to allow detection of RAS mutations by our procedure contain a mutation in codon 12 of the KRAS gene. Three mutations, G to T at the first base of the codon

Table III. Spectrum of KRAS codon 12/13 mutations

		pancreas n=28	colon n=60	lung n=14
K12 cys	G-T	36 *	12	43
ser	G-A	0	12	0
arg	G-C	4	0	0
val	G-T	28	16	21
asp	G-A	32	32	29
ala	G-C	0	7	7
K13 asp	G-A	0	21	0
	G-A	32	65	29
	G-T	64	28	64
	G-C	4	7	7

* indicated are percentages of the total number of
KRAS 12/13 mutations

and G to T or A at the second base, comprise 27 of the 28
mutations. The four samples that did not harbor a mutation in
codon 12 or 13 of the KRAS gene were further .analyzed for
mutations in codon 61 of the KRAS, NRAS and HRAS gene and for
mutations in codon 12 of the HRAS and codon 12 and 13 of the NRAS
gene. No mutations were detected.

To evaluate whether the type of mutations found in codon 12
of the KRAS gene in pancreatic adenocarcinomas exhibit a certain
specificity we have compared these mutations with KRAS mutations
occurring in colon tumors [4] and in lung adenocarcinomas [6]. As
shown in table III the predominant mutation in the pancreas are
G-T tranversions. The spectrum of mutations in the pancreas is
similar to the spectrum in lung, but differs from that in colon
tumors. In the latter tumors G-A transitions are more frequently
present.

DISCUSSION

Twenty-eight of the 30 patients with a tumor of the exocrine
pancreas harbor a mutated RAS gene in the tumor DNA and in all
cases the mutation is in codon 12 of the KRAS gene. These

results indicate that mutational activation of the KRAS gene might be a critical event in the development of pancreatic adenocarcinomas. In two cases RAS gene mutations could not be detected in the tumor DNA, although histologically these tumors do not differ from the tumors with a mutated RAS gene. It might be that in these cases the RAS genes incurred a mutation at a position which we did not screen. Alternatively, other defects with a similar effect as the mutational activation of the KRAS gene may have taken place. The relevance of activated RAS genes in the development of pancreatic tumors is further illustrated by experiments with transgenic mice which harbored a mutated HRAS gene under control of the elastase I promoter [12]. These mice developed carcinomas of all the fetal pancreas cells directly after the onset of elastase gene expression, indicating that the expression of a mutant HRAS gene is sufficient for the development of the tumor. This result, however, does not imply that for the development of spontaneous pancreatic tumors a single activated RAS gene is sufficient. In the latter case the RAS gene mutation arises in a single cell which, subsequently, has to grow out in the presence of the surrounding normal cells, whereas in the transgenic mice model all fetal pancreatic cells had the mutant RAS protein. A secondary event might be necessary to overcome possible inhibitory effects of normal cells. One of the events that may play an additional role in the development of human pancreatic tumors is the loss of the normal KRAS allele. This loss might reflect the loss of a putative tumor suppressor gene which is located in the vicinity of the KRAS gene. Alternatively, the normal KRAS allele may be lost by gene conversion resulting in two mutant alleles. Such a duplication can be important as has been shown for the HT1080 fibrosarcoma cell line. In this cell a single mutated NRAS allele, is not sufficient for the tumorigenicity of the cells [13]. Finally, the loss of the normal KRAS allele might prevent competition between the normal and the mutant KRAS protein. Evidence for such a competition is lacking, however.

The most striking observation is that all mutations occur in codon 12 of the KRAS gene. The specificity for the KRAS gene would be explained if only the KRAS and not the HRAS and NRAS is expressed in pancreatic cells. However, no information is

available to us about differential expression of these RAS genes. Alternatively, the various RAS proteins may have different functions and only activated KRAS proteins can induce pancreatic tumors. Although this explanation cannot be ruled out, there are no indications that activated RAS proteins have different properties. On the contrary, activated RAS proteins are considered to act autonomously in the activation of an effector molecule (protein). Recently, a putative effector protein, GAP, has been identified which binds to a domain identical in all three RAS proteins [14,15]. This indicates that all three RAS proteins might have the same effector molecule. A final explanation for the occurrence of unique mutations in the KRAS gene might be a different susceptibility to specific carcinogens.

The KRAS mutations found in pancreatic carcinomas are mainly G-T transversions at the first or second base and a G-A transition at the second base of codon 12. This mutation spectrum is similar to the spectrum found in lung carcinomas, but differs from that in colon tumors [Table III]. These differences might be due to tissue-specific factors, such as susceptibility to specific carcinogens or activity of certain DNA repair mechanisms, but could also reflect different chemical mutagens involved in the induction of these tumors. Indeed, from experiments with animal model systems it has been shown that different chemical mutagens can cause different mutations in the RAS genes [16]. With respect to pancreatic cancer, several chemical agents are used for the induction of tumors in a variety of animal models [17-19]. It is unknown, however, whether in these chemically induced pancreatic tumors mutated RAS genes are present. In humans, no direct causal relationship between any chemical agent and pancreatic carcinogenesis is found. From several epidemiological studies it appears that cigaret smoking is the only well established risk factor [20-22]. In this connection it is striking that the mutation spectrum found in pancreatic tumors is similar to that in lung tumors where smoking is an important factor in the induction of the KRAS mutation. The mutation spectra presented in this paper might help to identify chemical mutagens that specifically can induce mutations in the KRAS gene and, thus, represent risk factors for the induction of the major human cancers.

*To whom correspondence should be addressed

REFERENCES
1. Bos, J.L. (1988) Mut.Res. 195, 255-271.
2. Bos, J.L., Fearon, E.R., Hamilton, S.R., Verlaan-de Vries, M. and Vogelstein B. (1987) Nature 327, 293-297.
3. Forrester, K., Almoguera, C., Han, K., Grizzle, W.E. and Perucho, M. (1987) Nature 327, 298-303.
4. Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., Whyte, R., Smits, A.M.M. and Bos, J.L. (1988) New Engl.J.Med., in press.
5. Rodenhuis, S., Slebos, R.J.C., Boot, A.J.M., Evers, S.G., Mooi, W.J., Wagenaar, S.S., Van Bodegom, P.C. and Bos, J.L. (1988) Cancer Res. in press.
6. Bos, J.L., Verlaan-de Vries, M., Van der Eb, A.J., Jansen, J.W.G., Delwel, R., Lowenberg, B. and Colly, L.P. (1987) Blood 69, 1237-1241.
7. Farr, C.J., Saiki, R.K., Ehrlich, H.A., McCormick, F. and Marshall, C.J. (1988) Proc.Natl.Acad.Sci.USA 85, 1629-1633.
8. Almoquera, C., Shibata, D., Forrester, K., Martin, J., Arnheim, N. and Perucho, M. (1988) Cell 53, 549-554.
9. Bos, J.L., Verlaan-de Vries, M., Jansen, A.M., Veeneman, G.H., Van Boom, J.H. and Van der Eb. A.J. (1984) Nucl.Acids.Res. 12, 9155-9163.
10. Verlaan-de Vries, M., Bogaard, M.E., Van den Elst, H., Van Boom, J.H., Van der Eb, A.J. and Bos, J.L. (1986) Gene 50, 313-320.
11. Shibata, D., Arnheim N. and Martin J. (1988) J.Exp.Med. 167, 225-230.
12. Quaife, C.J., Pinkert, C.A., Ornitz, D.M., Palmiter, R.D. and Brinster, L. (1987) Cell 48, 1023-1034.
13. Paterson, H. Reeves, B., Brown, R., Hall, A., Furth, M., Bos, J.L. Jones, P. and Marshall, C.J. (1987) Cell 51, 803-812.
14. Adari, H., Lowy, D.R., Willumsen, B.M., Der, C.J. and McCormick, F. (1988) Science 240, 518-521.
15. Cales, C., Hancock, J.F., Marshall, C.J. and Hall, A. (1988) Nature 332, 548-551.
16. Guerrero, I and Pellicier A. (1987) Mut.Res. 185, 293-308.
17. Longnecker, D.S., Roebuck, B.D., Yager, J.D., Lilja, H.S. and Siegmund, B. (1981) Cancer, 47, 1562-1572.
18. Pour, P.M., Runge, R.G., Birt, D. et al. (1981) Cancer 47, 1573-1587.
19. Longnecker, D.S., Roebuck, B.D. Kuhlmann, E.T. and Curphey, T.J. (1985) JNCI, 74, 209-217.
20. Wynder, E.L., Mabuchi, K., Maruchi, N. and Fortner, J.G. (1973) JNCI, 50, 645-667.
21. Wynder, E.L. (1975) Cancer Res. 35, 2228-2233.
22. Mills, P.K., Beeson, W.L., Abbey, D.E., Fraser, G.E. and Philips, R.L. (1988) Cancer, 61, 2578-2585.